APPLICATION NOTE

Ploidy Analysis in Aquaculture

The aquaculture industry relies strongly on knowing the ploidy of its produce. Whether it's oysters, fish, or crustaceans, being able to select confidently which specimens you do or do not use for breeding directly influences the profitability, viability and sustainability of the industry. Fast, accurate and cost-efficient, modern flow cytometry is the answer of choice.

Background

Ploidy is the number of sets of chromosomes in a cell. Most fish, crustaceans and oysters occurring naturally contain diploid genetic material (even numbers of chromosomes, DNA), although spontaneous levels of polyploidy are also known to occur in nature. This is well documented in plants and fish species, such as the Siberian sturgeon (tetraploid). In general, organisms with an even number of chromosomes (e.g. diploids, tetraploids, etc.) are fertile, while those with odd numbers (i.e. triploids, pentaploids, etc.) are sterile.

Such modified, sterile breeds – generally triploids, are of great interest to aquaculture producers because:

a) they do not spend energy on reproduction and therefore grow faster than others
b) they have more chromosomes that support disease-resistant traits
c) they inhibit cross-breeding with wild type specimens and so limit the replacement of natural strains
d) their taste or other features are valuable because of different glycogen or fat content.
e) since they are sterile, they can sell larva or youngsters (fry, smolt, spat) every year to aquaculture farmers.

Identifying triploidy with flow cytometry versus other techniques

Techniques used for identifying polyploidy include DNA isolation, DNA amplification, DNA genotyping and molecular biology. All these techniques require specialised engineers, costly infrastructures and laboratories that limit their application. In comparison, flow cytometry is fast and inexpensive and delivers a more precise estimate of genome size, which is needed prior to DNA sequencing.

Using fluorescent dyes that bind to DNA molecules, flow cytometry can evaluate a cell's chromosome content by detecting the intensity of the fluorescence emitted by the dye-labelled nuclei. Depending on whether the number of chromosome sets are bisected or multiplied, living creatures can exhibit different characteristics.

Flow cytometry tests are well established in the plant breeding industry and basic research because of quantitative and qualitative advantages in industrial applications. FCM makes it possible to simultaneously record a wide range of parameters at ever increasing rates. In aquaculture, farming ponds or micro-organisms feeding reservoirs can be monitored simply and cost-effectively.

In this way, flow cytometry solutions deliver:

Accuracy: especially in comparison with visual methods used for oyster ploidy identification.
Speed: Protocols take less than 15 minutes and incubation time could be processed by batch of 10 samples.
Cost-efficiency: dedicated instrument simplified configurations, affordable reagents and consumables as well as simplicity of the laboratory settings make our solutions attractive in terms of investments.

Flow cytometry in practice

Sysmex has dedicated solutions for specific industries that depend on the configurations of the analysers. Alongside the quality of the light source, flow cytometry uses reagents to determine ploidy. For aquaculture, Sysmex has selected three unique reagents that can be used for products such as oysters, fish or crustaceans.

Table 1: *Selection of Sysmex solutions for ploidy analysis. Note: CyStain® reagent used depends on the flow cytometer configuration*

Reagent	Sysmex order codes	Suitable flow cytometer configurations
CyStain® UV Ploidy	05-5001	For UV excitation (UV LED or laser), for example ■ CyFlow® Space (CY-S-3001R_VS07) ■ CyFlow® Cube Ploidy Analyser (CY-S-3039_V1)
CyStain® UV Precise T	05-5003	For UV excitation (UV LED or laser), for example ■ CyFlow® Space (CY-S-3001R_VS07) ■ CyFlow® Cube Ploidy Analyser (CY-S-3039_V1)
CyStain® PI Absolute T	05-5023	Optimal for flow cytometer configurations with green (532) or suitable with blue (488 nm) laser excitations for example ■ CyFlow® Space (CY-S-3001R_VS08) ■ CyFlow® Cube Ploidy Analyser (CY-S-3039_V2)

Table 2: *Consumables needed for ploidy analysis.*

Material	Sysmex order codes
Petri dishes	04-2005
30 µm mesh CellTrics® filter	04-0042-2316
3.5 mL polystyrene sample tube	04-2000

Ploidy analysis in oyster samples (*Crassostrea gigas*)

Flow cytometry is the method of choice for ploidy analysis in oysters. Sysmex offers an all-round solution for analysing the different stages of oyster development: from larvae, to young oysters (spat) and adult oysters. The solution covers special reagents that are adapted specifically to the respective flow cytometer configurations within the Sysmex CyFlow® Series.

The right choice of sample material

The source of tissue for nuclei extraction depends on the stage of development.

- For larvae, several 60 micron-sized specimens should be harvested.
- For young oysters, one to five spat should be selected whereby tissue is extracted from the shell.
- For adult oysters a 5 mm^2 excision from the oyster mantle should be used.

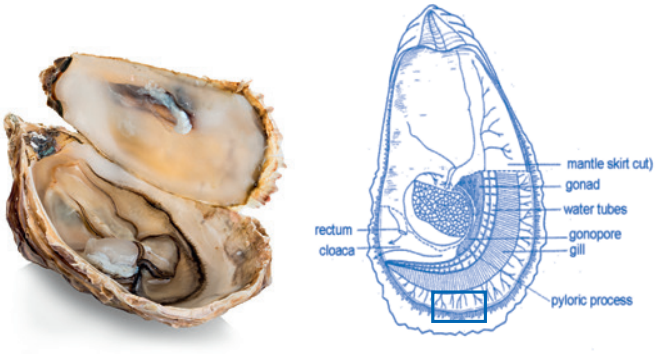


Figure 1: *Left-hand image:* Crassostrea gigas. *Right-hand image: the material suitable for analysis and size is indicated by the blue rectangle.*

Oyster nuclei extraction

Summary of the preparation of oyster material for ploidy analysis:

Pipet the extraction buffer, supplied with the CyStain® kit, into a Petri dish or other receptacle.

Note: Refer to the CyStain® package insert for quantities.

Place a piece of oyster tissue in the extraction buffer and carefully macerate with a razor blade.

Note: Be careful not to use a blunt razor blade (Gillette® Blue are recommended).

Tilt the petri dish. Using a pipette, draw the macerated sample into the pipette and then evacuate. Repeat 10 times to liberate nuclei from cellular debris.

Note: It is actually better to use a) a plastic Pasteur pipette, or b) an automatic pipette with cut tips. The narrow nozzle of a non-cut pipette tip could damage nuclei by shear forces.

Collect the maximum liquid sample with the same pipette and filter through a disposable 30 µm mesh CellTrics® filter into a 3.5 mL polystyrene sample tube.

Oyster nuclei staining

The process below is a proven, tried-and-tested staining procedure.

Add staining solution from CyStain® kit to the filtered sample.

Note: refer to the CyStain® package insert for quantities and incubation time.

After 30 seconds, re-suspend the sample (if necessary) and analyse by flow cytometry.

Note: Analysis by either using a CyFlow® Ploidy Analyser or CyFlow® Space (or CyFlow® Cube equipped with 532 nm or UV light source).

Oyster ploidy analysis

Below you will see a typical presentation of the ploidy level in the form of a histogram. It indicates the presence of the ploidy levels with respective peaks completed by an SSC histogram showing the distribution of inner granularity of the detected particles. The gates in plot FL2 against SSC display the detected 3n (triploids) and 2n (diploids), including the side scatter information. The results below were evaluated with the Sysmex CyFlow® Space software.

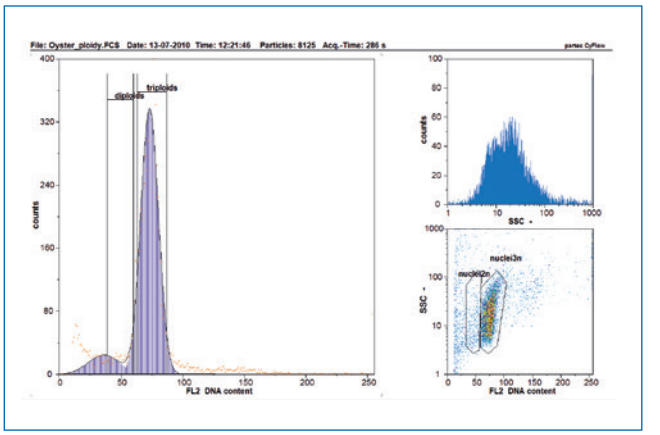


Figure 2: *Ploidy level analysed in oyster material with CyFlow® Space*

Flow cytometry and salmon samples (e. g. Atlantic salmon, *Salmo salar*)

Salmon farmers are particularly interested in the ploidy level of their stock. Triploid salmon are preferred as they grow larger and, since they are sterile, cannot breed with native salmon or trout populations should they escape the hatchery. However, triploid male salmon may display spawning behaviour that coaxes diploid females to spawn. It was found that over-ripening of eggs could lead to spontaneous triploid fish populations.

Ploidy in salmon is analysed at different developmental stages: eggs, larvae, smolt (salmon fry) and adults. The tissue source for analysis depends on the stage of development.

- For eggs, the whole egg is selected.
- For juvenile and adult fish, blood containing nucleated red blood cells is collected from the caudal vein and heparinised. Sometimes the material is fixed for 30 – 60 minutes with 4 % Paraformaldehyde. Alternatively, a 5 mm^2 piece of adult adipose fin clip can be harvested (see diagram below).

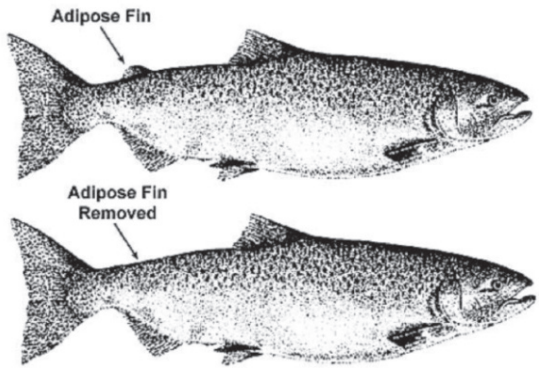


Figure 3: *Adipose fin is a dorsal fin without clear function and thought to be a left-over from the evolutionary past*

For the ploidy analysis in salmon, the Sysmex CyFlow® Ploidy Analyser or the Sysmex CyFlow® Space will indicate the ploidy levels.

Salmon nuclei extraction

Brief summary of a proven, tried-and-tested procedure

Pipet the extraction buffer, supplied with the CyStain® kit, into a Petri dish or other receptacle.

Note: Refer to the CyStain® package insert for quantities.

Place salmon tissue in the extraction buffer and carefully macerate with a razor blade.

Note: Be careful not to use a blunt razor blade. (Gillette® Blue are recommended).

Tilt the petri dish. Using a pipette, draw the macerated sample into the pipette and then evacuate. Repeat 10 times to liberate nuclei from cellular debris.

Note: It is actually better to use a) a plastic Pasteur pipette, or b) an automatic pipette with cut tips. The narrow nozzle of a non-cut pipette tip could damage nuclei by shear forces.

Collect the maximum liquid sample with the same pipette and filter through a disposable 30 µm mesh CellTrics® filter into a 3.5 mL polystyrene sample tube.

Salmon nuclei staining

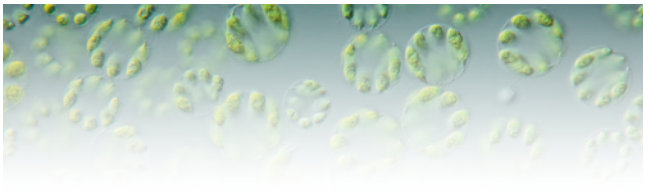
Summary of a simple staining process

Add staining solution from CyStain® kit to the filtered sample.

Note: refer to the CyStain® package insert for quantities and incubation time.

After 30 seconds, re-suspend the sample (if necessary) and analyse by flow cytometry.

Note: Analysis by either using a CyFlow® Ploidy Analyser or CyFlow® Space (or CyFlow® Cube equipped with 532 nm or UV light source).



Oysters feed on photosynthetic 'algae'

A few years ago, Sysmex recognised another flow cytometry-related application for oyster breeders who also count 'algae' in their ponds or water reservoirs that are used for feeding oysters.

Knowing the viability is useful and used by most French oyster breeders that have green laser excitation ploidy analysers such as the CyFlow® Space (CY-S-3001R_VS13). The viability test provides an indication of the algae's quality and is not available by standard microscopy.

This type of analysis discloses algae population information in terms of the:

- Absolute count
- Viability state, since algae cells change 'granularity' and fluorescence when they die

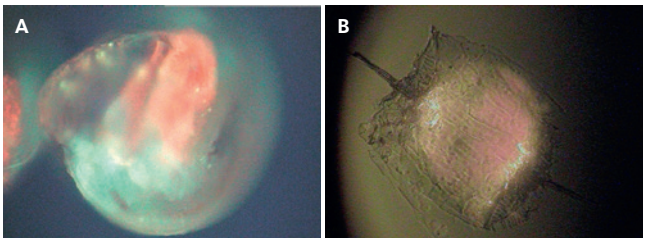


Figure 4A: *Oyster fed with algae (oyster in spat stage, microphotograph with CyScope® HP, 200 x magnification, 470 nm blue excitation*
Figure 4B: *Auto-fluorescent diatoms in seawater sample, taken with CyScope® HP, 400 x magnification, 470 nm blue excitation*

Counting unicellular algae using standard microscopy is time-consuming, imprecise and limited. Green laser ploidy analysers effectively detect those photosynthetic algae that belong to diatoms and other groups (*Isochrisys galbana 'affinis Tahiti'*, *Chaetoceros sp.*, *Skeletonema costatum*).

Auto-fluorescence from photosynthetic pigments delivers suitable, specific signals that are used as a parameter to identify algae species found in water tanks. Simply filter them through CellTrics® 30 µm and run samples displaying SSC and red emission fluorescence.

Analysing algae: *Chaetoceros sp.*

The Sysmex CyFlow® Space and Sysmex CyFlow® Ploidy Analyser are equipped with software that delivers analysis data of algae samples straight after running the samples. The plots and histograms present the number of counted algae (SSC-FSC plot) and the viability information is displayed in plots (FL2-SSC and FL3-SSC plots) or in the form of histograms.

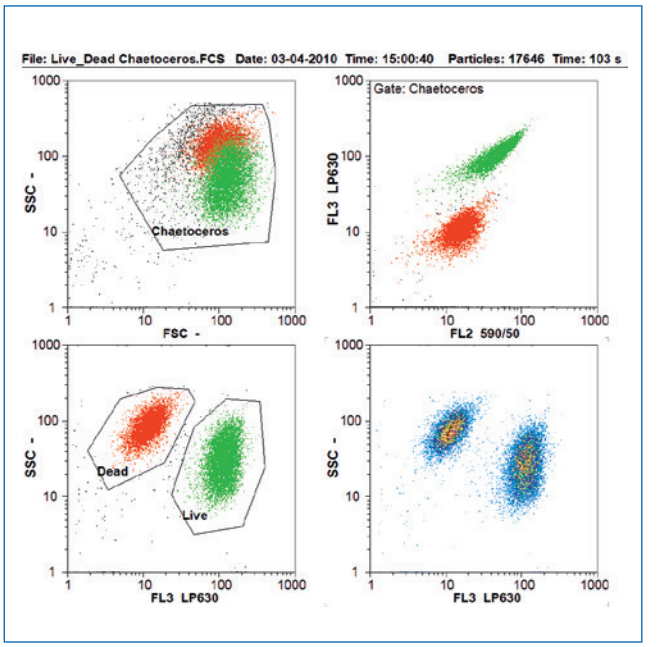


Figure 5: *Analysis of Chaetoceros sp on CyFlow® Space (CY-S-3001R_VS13)*

This procedure is applicable for any aquatic habitat, letting one characterise species' living conditions incredibly easily.

Summary of the simplicity of the analysis procedure:

Filter sample material taken from water tank using CellTrics® 30 µm.

Analyse on flow cytometer with SSC and red emission fluorescence.

Note: Analysis by either using a CyFlow® Space or CyFlow® Cube, for example on CyFlow® Space with blue and green lasers (CY-S-3001R_VS13).

Other species reported for polyploid induction in literature

Engineered and spontaneous triploidy (or hexaploidy) has been reported in a number of fish species: (scientific names by alphabetical order). Flow cytometry may therefore also be developed for these species specifically.

- Japanese eel (*Anguilla japonica*)
- Siberian sturgeon (*Acipenser baerii*) – tetraploid
- Sterlet (*Acipenser ruthenus*)
- White sturgeon (*Acipenser transmontanus*)
- Goldfish (*Carassius auratus*)
- Common carp (*Cyprinus carpio*)
- Atlantic cod (*Gadus morhua*)
- Turbot (*Scophtalmus maximus*)
- Coho salmon (*Oncorhynchus kisutch*)
- Rainbow trout (*Oncorhynchus mykiss*)
- Chinook salmon (*Oncorhynchus tshawytscha*)
- European catfish (*Silurus glanis*)
- Tench (*Tinca Tinca*)

Triploid induction has been adapted in a number of other group species (crustaceans, gastropods, echinoderms)

- Sea urchin (*Echinus esculentus*)
- Pacific abalone *(Haliotis discus*)
- Variously coloured abalone (*Haliotis diversicolor*)
- South African abalone (*Haliotis midae*)
- Blue mussel (*Mytilus edulis*)

Flow Cytometry as first choice for ploidy analysis

Ploidy analysis using Sysmex flow cytometry is well established. It can also be used for further research and industrial areas not mentioned here. Our local Sysmex representatives are happy to provide more information.

www.sysmex-flowcytometry.com

June 2017

Literature

[1] ***Liu S, Qin Q, Xiao J, Lu W, Shen J, Li W, Liu J, Duan W, Zhang C, Tao M, Zhao R, Yan J & Liu Y.*** *(2007): The formation of Polyploids Hybrids from different Subfamily Fish crossing and Evolutionary Significance. Genetics. 176, No 2, 1023 – 1024.*

[2] ***Zhang G, Wang Z, Chang Y, Song J, Ding J, Wang Y & Wang R.*** *(1998): Triploid induction in Pacific abalone Haliotis discus hannai Ino by 6-dimethylaminopurine and the performance of triploids juveniles. J. of Shellfish Research. 17, No 3, 783 – 788.*

Authorised representative: Sysmex Europe GmbH
Bornbarch 1, 22848 Norderstedt, Germany · Phone +49 40 52726-0 · Fax +49 40 52726-100 · info@sysmex-europe.com · **www.sysmex-europe.com**

Manufacturer: Sysmex Partec GmbH
Am Flugplatz 13, 02828 Görlitz, Germany · Phone +49 3581 8746-0 · Fax +49 3581 8746-70 · info@sysmex-partec.com · **www.sysmex-partec.com**

Sysmex Corporation
1-5-1 Wakinohama-Kaigandori, Chuo-ku, Kobe 651-0073, Japan · Phone +81 78 265-0500 · Fax +81 78 265-0524 · **www.sysmex.co.jp**

You will find your local Sysmex Partec representative's address under www.sysmex-partec.com

ZE001707.EN.N.06/17